

C3a Is Required for the Production of CXC Chemokines by Tubular Epithelial Cells after Renal Ischemia/Reperfusion¹

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The complement system is one of the major ways by which the body detects injury to self cells, and the alternative pathway of complement is rapidly activated within the tubulointerstitium after renal ischemia/reperfusion (I/R). In the current study, we investigate the hypothesis that recognition of tubular injury by the complement system is a major mechanism by which the systemic inflammatory response is initiated. Gene array analysis of mouse kidney following I/R initially identified MIP-2 (CXCL2) and keratinocyte-derived chemokine (KC or CXCL1) as factors that are produced in a complement-dependent fashion. Using *in situ* hybridization, we next demonstrated that these factors are expressed in tubular epithelial cells of postischemic kidneys. Mouse proximal tubular epithelial cells (PTECs) in culture were then exposed to an intact alternative pathway and were found to rapidly produce both chemokines. Selective antagonism of the C3a receptor significantly attenuated production of MIP-2 and KC by PTECs, whereas C5a receptor antagonism and prevention of membrane attack complex (MAC) formation did not have a significant effect. Treatment of PTECs with an NF- κ B inhibitor also prevented full expression of these factors in response to an intact alternative pathway. In summary, alternative pathway activation after renal I/R induces production of MIP-2 and KC by PTECs. This innate immune system thereby recognizes hypoxic injury and triggers a systemic inflammatory response through the generation of C3a and subsequent activation of the NF- κ B system. *The Journal of Immunology*, 2007, 178: 1819–1828.

Ischemia/reperfusion (I/R)³ of the kidney is followed by a robust inflammatory reaction in the renal tubulointerstitium (1). Studies in rodents have suggested that many components of the innate immune system contribute to renal injury after I/R, including the complement system (2–6), neutrophils (7), and monocytes (8). Cells of the cognate immune system have also been implicated in ischemic acute renal failure (ARF), including T cells (8–10), and possibly B cells (11, 12). Human acute tubular necrosis is characterized by engagement of many of the same inflammatory factors seen in animal models (13–16). Inflammatory cytokines and chemokines are generated in the ischemic kidney (7, 17) and likely orchestrate this broad inflammatory response. Generation of these signals may be a direct response of cells to hypoxia, but there is evidence that innate immune systems such as the TLRs (18) and the complement system recognize injury to the PTECs and elicit production of these factors (5).

The complement system is a rapid and efficient system for the detection of invasive organisms or aseptic injury to host cells (19), and we have reported that I/R causes the proximal tubular epithelial cell to change from a complement inhibitory to a complement activating phenotype (20). For example, hypoxic PTECs synthesize C3 and express decreased complement inhibitory proteins on their basal surface (20). Once activated, the complement system generates the C3a and C5a anaphylatoxins, and systemic levels of C3a are measurably increased in mice after renal I/R (21). Complement activation fragments may induce cells to produce a number of cytokines and chemokines (6, 22, 23). The complement system may therefore “recognize” injury to the hypoxic PTEC and signal cells to elaborate chemokines, thereby initiating an inflammatory response to injury.

To determine whether complement activation in the postischemic kidney is associated with chemokine production, we performed gene array analysis on tissue from wild-type and factor B deficient mice. We found that mRNA for the C-X-C chemokines MIP-2 and KC are increased in the kidney after I/R, but that production of these factors is attenuated in factor B deficient mice. Elaboration of chemokines within the postischemic kidney may be an important mechanism by which complement activation initiates inflammation and causes renal injury. In the current study we examine mechanisms by which complement activation in the renal tubulointerstitium triggers the production of these chemokines.

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³ Abbreviations used in this paper: I/R, ischemia/reperfusion; ARF, acute renal failure; KC, keratinocyte-derived chemokine; PTEC, proximal tubular epithelial cell; MAC, membrane attack complex.

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Materials and Methods

Animals

Mice deficient in factor B were generated as previously described (24), and were backcrossed onto C57BL/6J mice (Jackson Laboratories) for seven generations. The *fB*^{-/-} mice cannot activate complement by the alternative pathway but have intact classical and lectin pathway activation mechanisms. Eight- to ten-week-old *fB*^{+/+} and *fB*^{-/-} mice were used for the *in vivo* experiments.

Renal I/R protocol

Mice were anesthetized with 300 μ l of 2,2,2-tribromoethanol (Sigma-Aldrich) injected i.p., and they were placed on a heating pad to maintain their body temperature during surgery. Laparotomies were then performed, and the renal pedicles were located and isolated by blunt dissection. The pedicles were clamped with surgical clips (Miltex Instrument), and occlusion of blood flow was confirmed by visual inspection of the kidneys. The clamps were left in place for 24 min and then released. This time of ischemia was chosen to obtain a reversible model of ischemic ARF with a minimum of vascular thrombosis, and to avoid animal mortality. The kinetics of complement activation in this model have been previously described (20). The kidneys were observed for \sim 1 min to ensure blood reflow, then fascia and skin were sutured with 4–0 silk (U.S. Surgical). Sham surgery was performed in an identical fashion, except that the renal pedicles were not clamped. The mice were volume resuscitated with 0.5 ml of normal saline injected subcutaneously and kept in an incubator at 29°C to maintain body temperature until the time of sacrifice. After 2 or 8 h of reperfusion the mice were anesthetized, and blood was obtained by cardiac puncture. Laparotomy was performed and the kidneys were harvested. The study protocol was approved by the University of Colorado Health Sciences Center Animal Care and Use Committee.

Gene array analysis

Sham-treated and postischemic kidneys were obtained from mice after ischemia and 2 or 8 h of reperfusion. RNA was isolated from sagittal kidney sections using TRIzol reagent (Invitrogen Life Technologies) according to the manufacturer's instructions. For both time points, two samples each were included from sham-treated wild-type mice, postischemic wild-type mice, and postischemic *fB*^{-/-} mice, for a total of 12 samples. Microarrays were performed with the murine genome expression set 430 (Affymetrix) as previously described (25). In brief, the quality of the RNA was confirmed by evaluation on an Agilent 2100 Bioanalyzer. Next, 10 μ g of total RNA was used to generate double-stranded cDNA and biotin-labeled anti-sense cRNA. The cRNA was fragmented and hybridized to the array, washed and stained, and results were measured using a GeneArray scanner (Affymetrix). Comparison analyses were performed with Affymetrix Microarray Suite (version 5.0), and the hybridization intensity data from the arrays for the different groups at each time point were examined to determine the change in transcription for each evaluated gene. The results of this analysis were then sorted and analyzed using Microsoft Excel. Data were compared to determine which genes showed greater than a 4-fold increase in transcription after ischemia but showed <50% transcription in the ischemic *fB*^{-/-} mice compared with the ischemic wild-type mice. Of the four possible comparisons between each group, we also determined how many of these comparisons demonstrated the increase or decrease in gene expression.

Quantitative real-time PCR

RNA was isolated from kidneys as described above. For in vitro experiments, RNA was isolated directly from the culture plates using TRIzol. cDNA was generated from 1 μ g of RNA using murine leukemia virus reverse transcriptase (Applied Biosystems). Appropriate primers were designed using Beacon Designer software (PREMIER Biosoft International), and quantitative real-time PCR for MIP-2 and KC was performed using the designated primers (Table I). Samples were prepared using iQ Sybergreen Supermix (Bio-Rad) and the iCycler iQ detection system (Bio-Rad). Triplicate reactions for each sample were performed using the target primers and using cyclophilin primers to provide an internal loading control. After expressing the target gene as a function of cyclophilin expression, the

relative expression of the target genes were expressed normalized to a sample from a wild-type sham-treated animal. This sample was included in all reactions to normalize and compare data.

MIP-2 and KC protein assay

Protein levels of MIP-2 and KC were measured using commercially available quantitative sandwich enzyme immunoassays according to the manufacturer's instructions (R&D Systems). For cell culture supernatants, 50 μ l of the supernatant was used in the assay for each sample. For serum measurements, the samples were diluted 1:1 and then used in the reaction.

In situ hybridization

For in situ hybridization, sagittal sections the kidneys were snap frozen in OCT compound (Sakura Finetek) and 16- μ m sections were made. Sections from wild-type and *fB*^{-/-} kidneys were processed in parallel to allow direct comparison. The sections were fixed for 15 min in cold 4% paraformaldehyde. The sections were then hybridized with digoxin-labeled anti-sense probes to mouse MIP-2 and KC (Table I) according to the manufacturer's instructions (Genedetect). The bound probe was detected using alkaline phosphatase-labeled anti-digoxigenin Fab and NBT/BCIP tablets (Roche Applied Science). Digoxin-labeled sense probe to both targets was used to confirm the specificity of the binding. Slides were counterstained with methyl green (Vector Laboratories). Images were obtained using a Nikon eclipse E400 microscope with Spot version 3.5.2 software (Diagnostic Instruments).

In vitro assay of PTEC response to complement activation

To evaluate the direct response of PTECs to complement activation without other cells present, immortalized PTECs were used. The BUMPT cell line is a mouse PTEC line that was established from the Immortomouse (26) and was generously provided to us by John Schwartz (Boston University). Cells were grown to confluence in 24-well plates with DMEM medium supplemented with 10% FBS, penicillin-streptomycin, and 0.2 U/ml IFN- γ (Peprotech). The IFN- γ in this medium permits expression of the H-2K^b-tsA58 transgene in these cells (26). After the cells reached confluence they were changed to 1:1 DMEM/Ham's F12 supplemented with 5 mg/L transferrin (Invitrogen Life Technologies), 50 nM hydrocortisone (Sigma-Aldrich), and 5 mg/L insulin for 2 days. Expression of the transgene is almost completely suppressed under these conditions (26). As a source of complement components, pooled serum from C57BL/6J mice was added to the medium for a final concentration of 10%. To accommodate for variation in the response to serum batches, direct comparisons are only made between cells treated side by side with the same serum batch. We have previously demonstrated that under these conditions the cells do not undergo a significant degree of spontaneous complement-mediated lysis (21), and pilot experiments demonstrated that when exposed to this concentration of serum, the transcription of the target genes increased after 1 h. To explore the pathways by which serum elicits production of the chemokines by these cells, the alternative pathway was blocked in the serum using the highly inhibitory mouse anti-mouse factor B mAb 1379 (0.5 μ g/ μ l) (27) in medium containing 10% serum. To block specific complement activation fragments, the cells were treated with a small molecule (5 or 50 μ M of SB290157; Calbiochem). This agent has been used as a specific C3a receptor antagonist (28), although in some model systems it has been shown to have C3a agonist activity that mirrors that seen with C3a (29). We also used a C5a receptor antagonist [5 or 50 μ M of cyclic hexapeptide AcF[OPdChaWR] (30)] or 50 μ g/ml a mAb to C5 which prevents C5a and MAC formation [mAb BB5.1 (31)]. Experiments were also performed using purified C5a (1 μ g/ml; Sigma-Aldrich) and serum

Table I. Primers and probes

Real-time PCR primers	
MIP-2 forward	5'-ACC AAC CAC CAG GCT ACA G-3'
MIP-2 reverse	5'-GCG TCA CAC TCA AGC TCT-3'
KC forward	5'-GGC TGG GAT TCA CCT CAA GAA C-3'
KC reverse	5'-TGT GGC TAT GAC TTC GGT TTG G-3'
Cyclophilin forward	5'-TGG AGA GCA CCA AGA CAG ACA-3'
Cyclophilin reverse	5'-TGC CGG AGT CGA CAA TGA T-3'
In situ hybridization probes	
MIP-2 antisense	5'-GCC TTG CCT TTG TTC AGT ATC TTT TGG ATG ATT TTC TGA ACC AGG GGG-3'
KC antisense	5'-CTT CAG GGT CAA GGC AAG CCT CGC GAC CAT TCT TGA GTG TGG CTA TGA-3'

from $C6^{-/-}$ mice (32). To determine whether the MIP-2 and KC that is released by the cells is synthesized de novo or whether it involves the release of preformed protein, cells were exposed to cycloheximide (Sigma-Aldrich) 20 $\mu\text{g}/\text{ml}$ for 2 h before exposure to the serum. To examine the signal transduction pathways by which complement activation induces transcription of these factors, the cells were incubated with inhibitors of ERK1/2 (50 μM PD98059; Calbiochem), JNK (10 μM SP600125; Calbiochem), p38 (10 μM SB203580; Calbiochem), and NF- κB (10 μM Bay11-7082; Calbiochem).

Statistical analyses

Multiple group comparisons were performed using ANOVA with posttest according to Newman-Keuls. A value of $p < 0.05$ was considered statistically significant. Results are reported as mean \pm SEM.

Results

Transcriptional response of the kidney to complement activation after renal I/R

Gene array analysis was performed on cDNA made from the kidneys from $fB^{+/+}$ and $fB^{-/-}$ mice subjected to I/R or sham treatment, and the transcription of chemokines was specifically examined (Fig. 1). When stringent criteria were applied to the data, only the gene for MIP-2 (Fig. 1A) at the 8 h time-point was increased in a complement-dependent fashion. The gene for KC was increased >28 -fold after I/R (Fig. 1B), although levels in the $fB^{-/-}$

mice were reduced by slightly $<50\%$. Other chemokines, such as MCP-1 (Fig. 1C) and CXCL14 (Fig. 1D), demonstrated increased expression after I/R. The $fB^{-/-}$ mice did not both demonstrate lower expression than the wild-type mice, however, and we did not evaluate them further (see methods). Ten other genes appeared to increase in a complement-dependent fashion using our selection criteria. As these other genes did not encode chemokines, their expression was not examined further in the current study.

Synthesis of MIP-2 and KC within the kidney is increased after I/R in a complement-dependent fashion

To confirm the gene array results described above, quantitative real time PCR for MIP-2 and KC was performed on kidneys from $fB^{+/+}$ and $fB^{-/-}$ mice subjected to I/R or sham treatment (Fig. 2). Transcription of both genes was significantly increased in the wild-type mice after ischemia and 8 h of reperfusion compared with sham-treated controls. In the $fB^{-/-}$ mice, however, levels of mRNA for both genes were significantly lower than in the wild-type mice subjected to I/R. These findings suggest that production of both MIP-2 and KC after renal I/R occurs downstream of complement activation, and that lack of a functional alternative pathway prevents their generation after I/R.

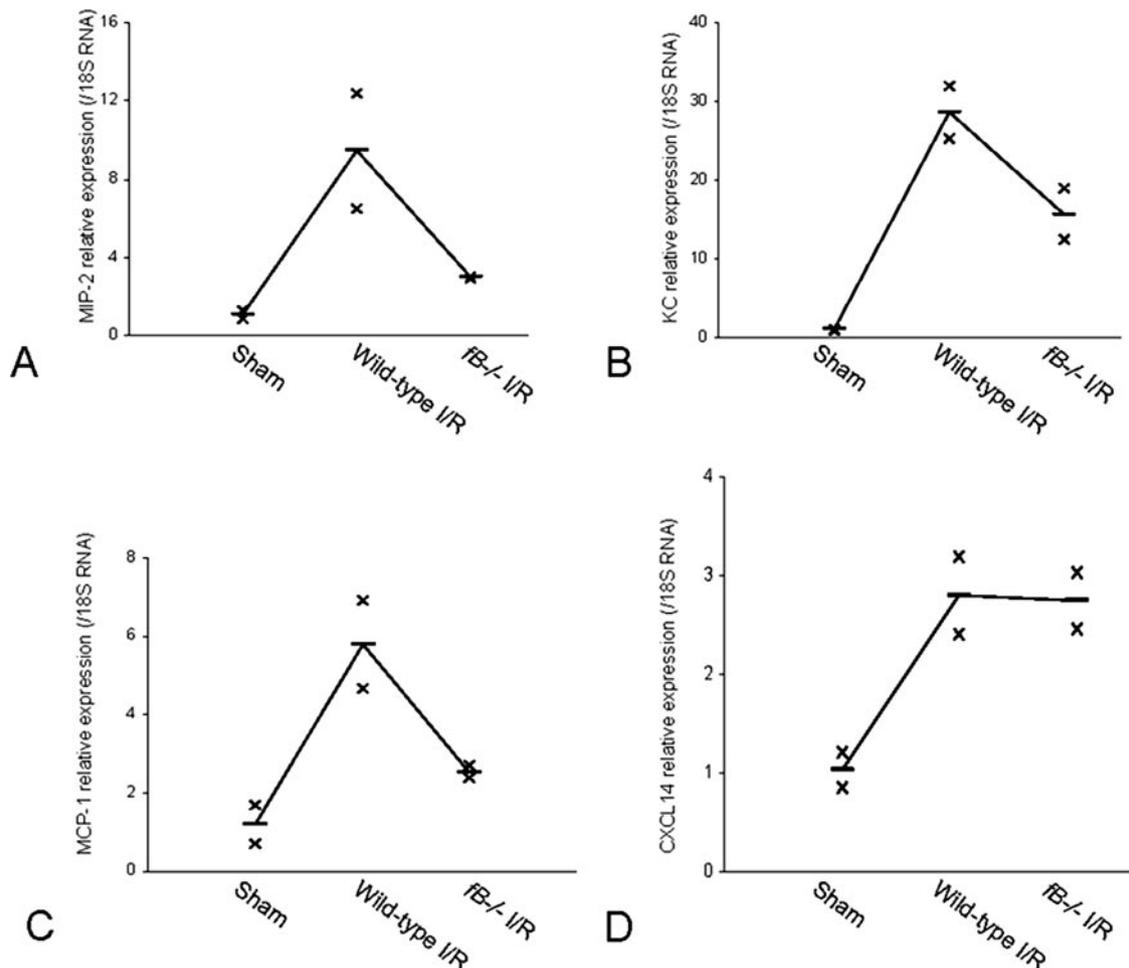
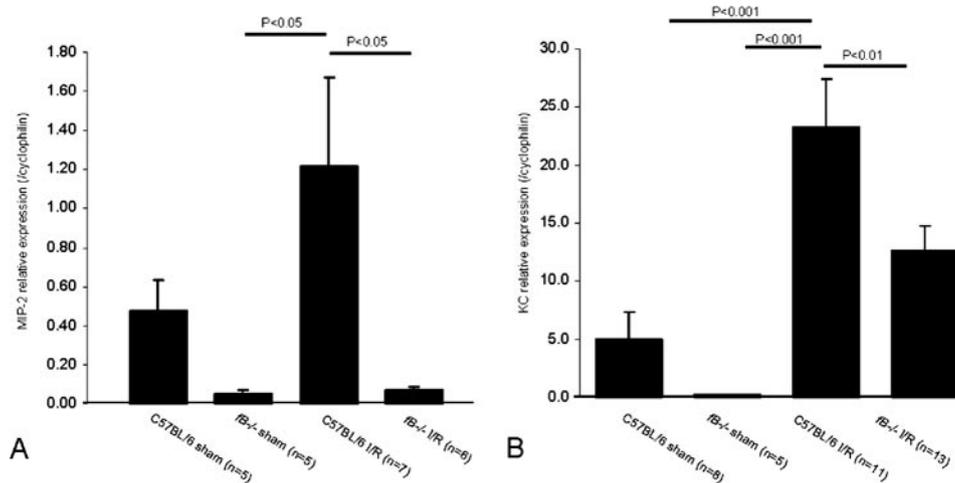


FIGURE 1. Chemokine expression in the kidneys of wild-type and $fB^{-/-}$ mice after I/R. Gene array analysis was performed to measure mRNA expression of chemokines in the kidneys of wild-type mice subjected to sham treatment or renal I/R, and $fB^{-/-}$ mice subjected to renal I/R. The relative expression within the kidney of several chemokines increased after ischemia and 8 h of reperfusion and are shown in this figure. For these genes, the relative values for each sample (x) and the mean for each group (—) are shown. The increase in MIP-2 (A) and KC (B) was attenuated in both $fB^{-/-}$ mice relative to the wild-type mice subjected to I/R.

FIGURE 2. MIP-2 and KC mRNA increase in the kidney after I/R in a complement-dependent fashion. Quantitative real-time PCR was performed on cDNA made from kidneys subjected to sham treatment or ischemia and 8 h of reperfusion. The relative mRNA expression levels of the chemokines were normalized to cyclophilin mRNA expression. Transcription of both chemokines was significantly increased after I/R in wild-type mice, but were significantly lower in $fB^{-/-}$ mice subjected to I/R.



MIP-2 and KC are produced by tubular epithelial cells after I/R

Immunofluorescence microscopy has demonstrated that C3 deposition in the kidney after I/R occurs primarily along the injured tubules of the outer medulla (2). Complement activation may induce production of chemokines by direct stimulation of cells upon which complement activation fragments are deposited or by generation of fragments such as C3a and C5a that may diffuse away and act on nearby cells. It is also possible that synthesis is performed by inflammatory cells that traffic to the kidney and/or are activated in response to complement activation fragments. We performed in situ hybridization to determine which cell types were responsible for chemokine production in the kidney. Kidney sections from $fB^{+/+}$ and $fB^{-/-}$ mice after ischemia or sham treatment and 8 h of reperfusion were evaluated (Figs. 3 and 4). In sham-treated mice, a small amount of MIP-2 mRNA was detectable in the glomeruli (Fig. 3A) and in the tubules (Fig. 3B). After I/R, MIP-2 mRNA was increased in the tubules, particularly in the outer medulla (Fig. 3F). MIP-2 mRNA was virtually undetectable in the sham-treated $fB^{-/-}$ mice (Fig. 3, C and D), and levels appeared slightly greater in the glomeruli and tubules of $fB^{-/-}$ kidneys subjected to I/R (Fig. 3, G and H). The specificity of the signal was confirmed by hybridizing sense probe to the tissue, and no signal was visualized (not shown).

Message for KC was not readily detectable in the glomeruli or tubules of sham-treated mice (Fig. 4, A–D). A small amount of KC mRNA was detectable in the glomeruli of C57BL/6 mice subjected to ischemia (Fig. 4E), and signal was much more evident in cells of the tubulointerstitium. This was heaviest in the outer medulla. No clear signal was seen in $fB^{-/-}$ mice after either sham treatment or ischemia (Fig. 4, G and H).

We have previously examined infiltration of the kidneys by neutrophils after I/R (2). We found that wild-type mice have a significant increase in the number of neutrophils seen in the outer stripe of the outer medulla but $fB^{-/-}$ mice had very few detectable neutrophils. In time-course experiments we did not see significant numbers of neutrophils within the first 8 h of reperfusion, the period when chemokine expression peaks. Thus, expression of these factors in the outer medulla seems to occur rapidly after I/R and is followed sequentially by the influx of neutrophils into this region.

Systemic and urine levels of MIP-2 are increased by complement activation within the kidney after renal I/R

To assess whether increased transcription of MIP-2 results in increased protein production, serum from mice subjected to ischemia or sham treatment was assayed for MIP-2 by ELISA (Fig. 5A).

Levels of MIP-2 were significantly increased in wild-type mice after ischemia and 8 h of reperfusion. In contrast, levels of MIP-2 in $fB^{-/-}$ mice were not significantly greater than those seen in sham-treated controls. Levels of MIP-2 in the urine of wild-type mice were also significantly higher after I/R (Fig. 5B), but did not increase in $fB^{-/-}$ mice after I/R. Levels of KC could not be determined in these samples because the assay was altered by even small amounts of hemolysis.

Alternative pathway activation induces PTECs in culture to produce MIP-2 and KC

As shown above, complement activation in the renal tubulointerstitium after I/R is associated with production of MIP-2 and KC by PTECs. To evaluate whether complement activation directly causes PTECs to elaborate these factors or whether it requires other ischemia related factors or other cell types, we examined the effects of complement activation on PTECs in culture. Exposure of the cells to serum with an intact alternative pathway for 1 h induced significant increases in the mRNA for both MIP-2 and KC (Fig. 6). Levels of both chemokines as measured by ELISA were also significantly increased in the supernatants of these cells. Selective inhibition of the alternative pathway with mAb 1379 prevented the increase in mRNA and protein levels for both chemokines, confirming that this is a downstream effect of alternative pathway activation. A similar reduction in chemokine production was seen when serum from $fB^{-/-}$ mice was applied to the cells (data not shown), although these mice may have altered levels of other complement components that could confound direct comparisons.

Production of MIP-2 and KC by PTECs requires signaling through the C3a receptor

We repeated the in vitro experiment after treating the cells with different concentrations of inhibitors of the C3a and C5a receptors (Fig. 7). At 50 μ M the C3a receptor antagonist SB290157 reduced production of MIP-2 and KC, so this concentration of receptor antagonist was directly compared with 1379 and found to be similarly efficacious at attenuating production (Fig. 7C). This agent has also been reported to be a C3a receptor agonist in some systems, mimicking the effect of C3a (29). This agent does not cause production of the MIP-2 and KC in our system (Fig. 7E), however, and it prevents complement mediated production of these chemokines, indicating that this effect is due to antagonism of the receptor. Purified C3a (Calbiochem no.204881) at concentrations up to 1.66 μ M did not elicit production of these chemokines (data not

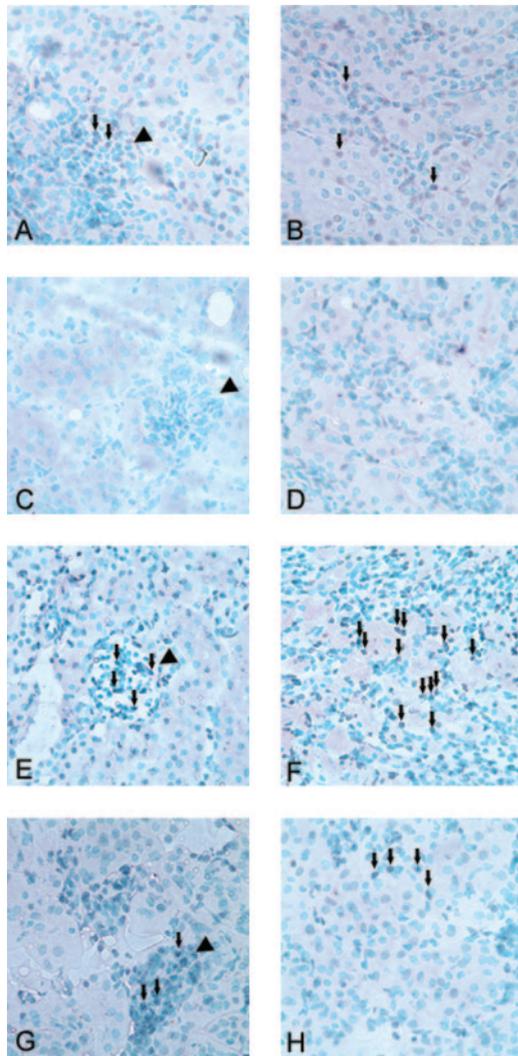


FIGURE 3. In situ hybridization for MIP-2 mRNA. In situ hybridization was performed to detect mRNA for MIP-2 in kidneys after sham treatment or ischemia followed by 8 h of reperfusion and slides were counterstained with methyl green. Arrowheads indicate glomeruli. Arrows indicate examples of cells with positive signal for MIP-2. A small amount of signal was evident in the glomeruli (A) and tubules of the outer medulla (B) of C57BL/6 mice subjected to sham treatment, whereas no signal was evident in sham-treated $jB^{-/-}$ mice. Increased message was seen in the glomeruli (E) and tubules (F) of C57BL/6 mice after I/R, and to a much lesser degree in $jB^{-/-}$ mice (G and H). Original magnification, $\times 400$. Representative images are shown; $n = 5$ for each experimental group.

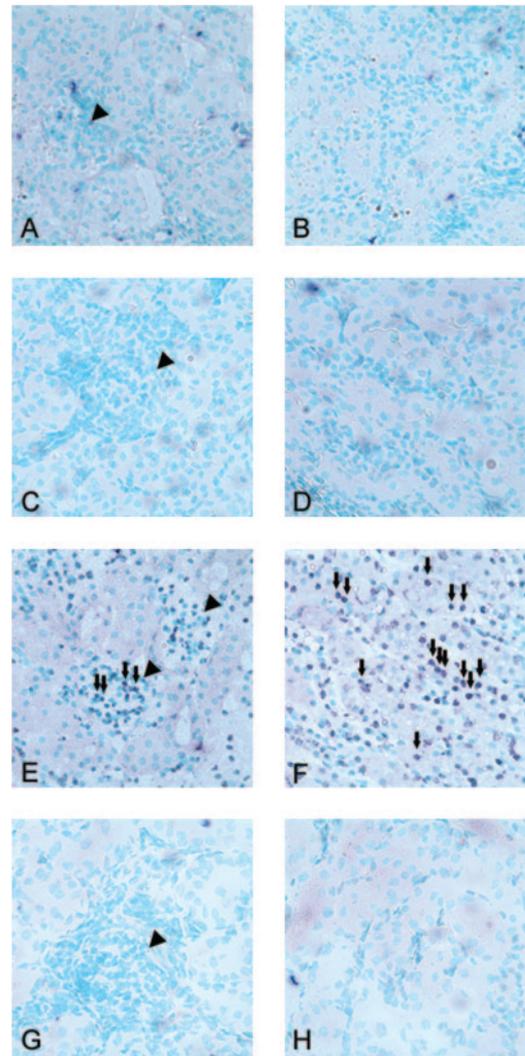


FIGURE 4. In situ hybridization for KC mRNA. In situ hybridization was performed to detect mRNA for KC in kidneys after sham treatment or ischemia followed by 8 h of reperfusion and slides were counterstained with methyl green. Arrowheads indicate glomeruli. Arrows indicate tubular epithelial cells. Little signal was seen in the glomeruli or tubules of sham-treated C57BL/6 (A and B) or $jB^{-/-}$ mice (C and D). Increased message was seen in the glomeruli (E) and tubules (F) of C57BL/6 mice after I/R, predominantly at the cortico-medullary junction. Very little staining was seen in the glomeruli (G) or tubules (H) of $jB^{-/-}$ mice subjected to I/R. Staining appeared much less intense in $jB^{-/-}$ mice. Original magnification, $\times 400$ except where $\times 100$ is indicated. Representative images are shown; $n = 5$ for each experimental group.

shown), suggesting that a cofactor present in serum is necessary for C3a mediated production.

The C5a receptor antagonist and pretreatment of the serum with an anti-C5 Ab, in contrast, did not affect chemokine production. Isolated C5a did, however, induce the PTECs to secrete KC but not MIP-2. This suggests that C5a can signal for KC production, but that there are parallel pathways which are activated even in the absence of C5a. Experiments using $C6^{-/-}$ serum or serum pretreated with the anti-C5 Ab were used to assess whether MAC deposition is an important stimulus for chemokine production by PTECs. In both instances, chemokine production was comparable to that seen using wild-type serum. Thus, complement-induced MIP-2 and KC production by PTECs appears to be mediated by C3a.

PTECs were also exposed to serum after pretreatment with cycloheximide at 20 $\mu\text{g/ml}$ (Fig. 8). Treatment with cycloheximide prevented the increase of these chemokines in the supernatants of cells exposed to serum, demonstrating that the release of these factors is due to de novo synthesis and not due to release of preformed protein from injured cells.

Complement-induced production of MIP-2 and KC by PTECs is mediated by NF- κ B

To explore which intracellular signal transduction pathways trigger the production of MIP-2 and KC by PTECs in response to complement activation, cells were treated with inhibitors of ERK 1/2, JNK, P38, and the NF- κ B pathway (Fig. 9). Of these pathways, only NF- κ B inhibition significantly attenuated the production

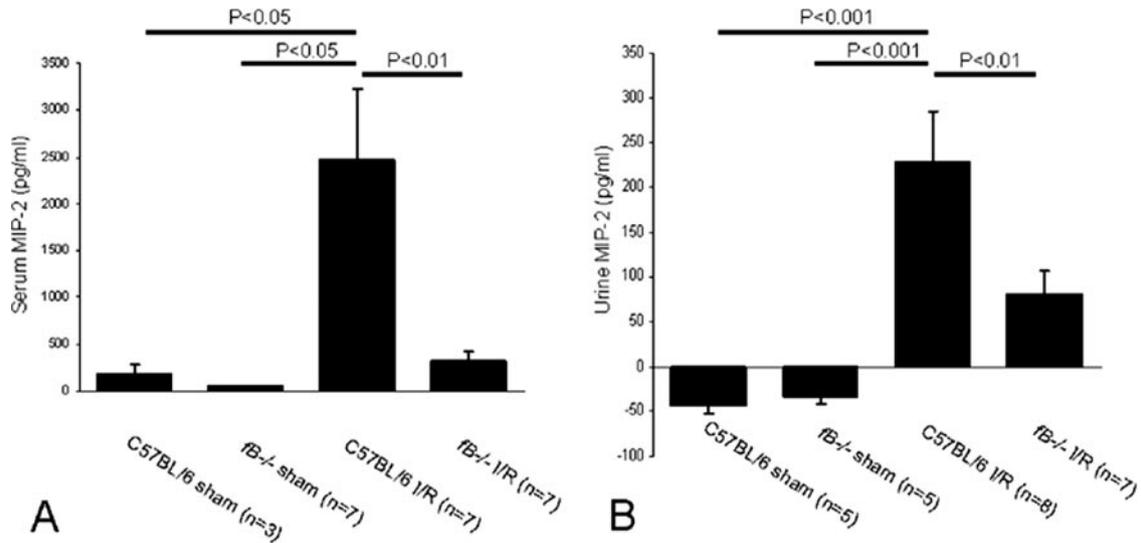


FIGURE 5. Serum and urine MIP-2 is increased after I/R. Serum and urine were obtained from wild-type and $fB^{-/-}$ mice subjected to ischemia and 8 h of reperfusion. Levels of MIP-2 were measured by ELISA, and values were determined by comparison with a standard curve. For serum (A) and urine (B) samples, MIP-2 levels were significantly increased in wild-type mice subjected to ischemia, and levels were significantly lower in $fB^{-/-}$ mice after I/R when compared with samples from wild-type mice subjected to I/R. The negative values obtained in urine from sham-treated mice may reflect interference of the assay by urine.

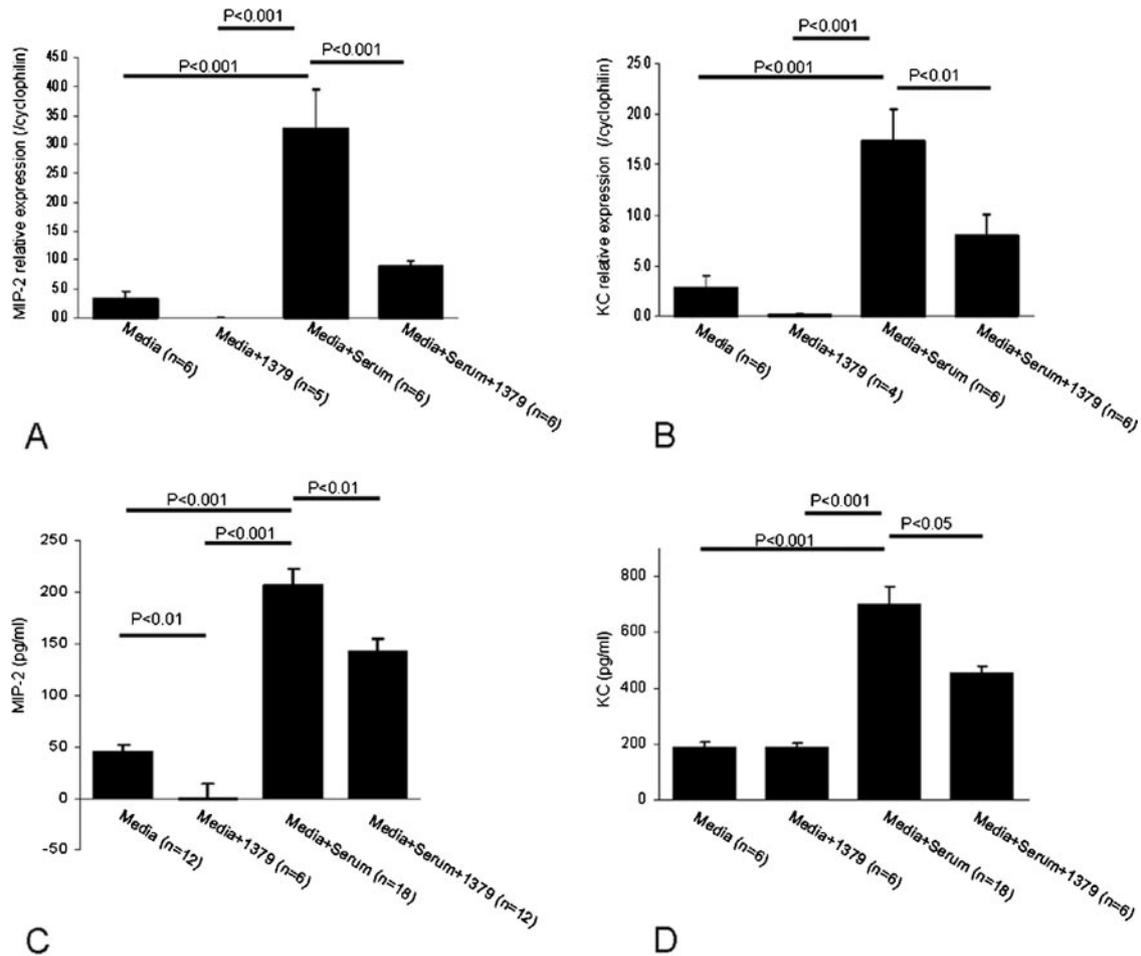


FIGURE 6. Epithelial cells in culture produce MIP-2 and KC in response to an intact alternative pathway. Murine PTECs were grown to confluence and exposed to fresh serum. After 1 h, mRNA for both chemokines was measured by quantitative real-time PCR (A and B), and chemokine levels in the supernatants were measured by ELISA (C and D). In the presence of an intact alternative pathway, mRNA and protein for both chemokines increased significantly. Inhibition of the alternative pathway with 1379 (an inhibitory mAb to mouse factor B) prevented production of either protein by the cells. Protein concentrations were determined by comparison with a standard curve.

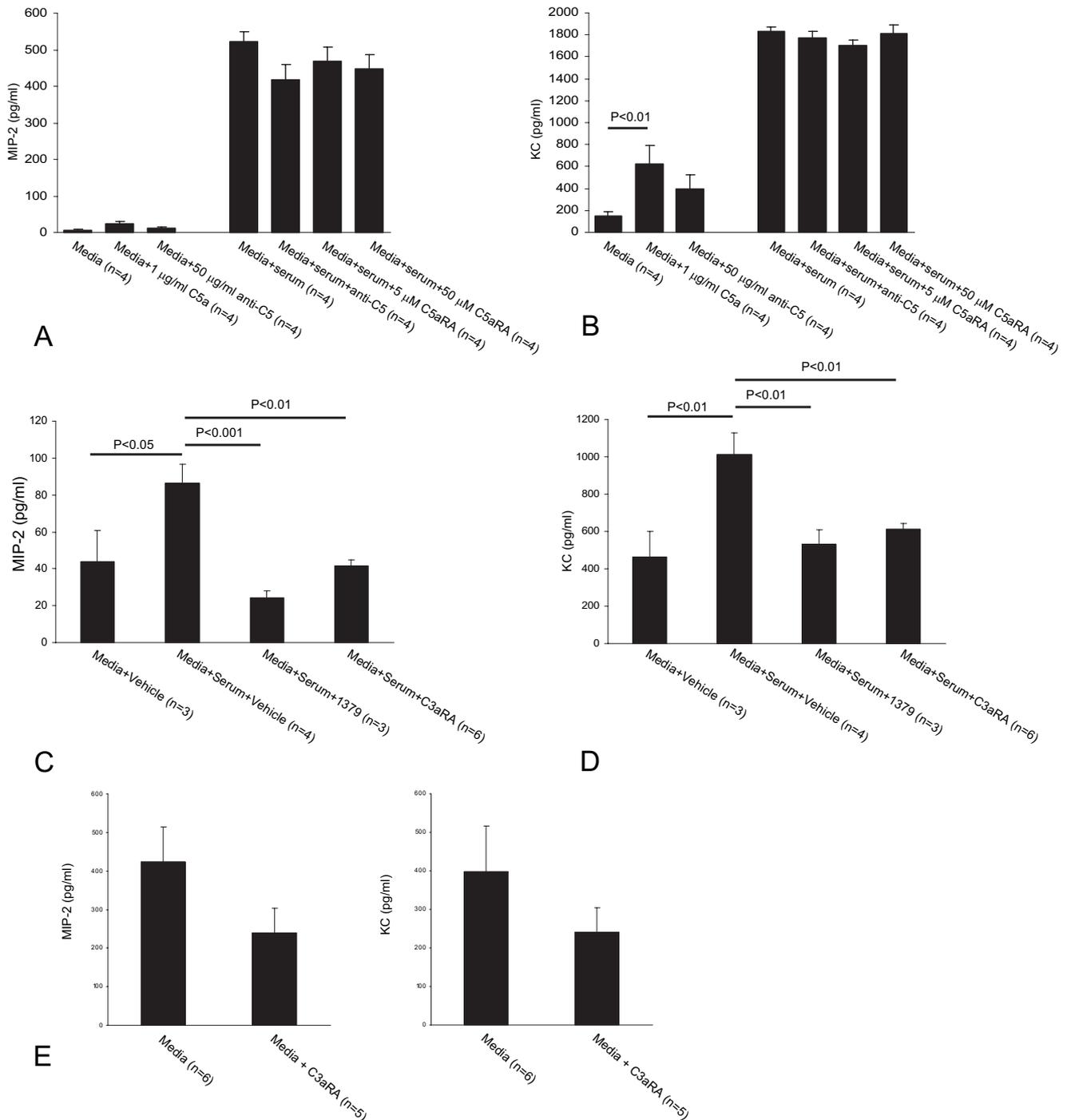


FIGURE 7. C3a mediates production of MIP-2 and KC by tubular epithelial cells. Tubular epithelial cells were exposed to fresh serum after pretreatment for 1 h with inhibitors of the C3a receptor and C5a receptor. Cells were also exposed to serum that was incubated with an inhibitory mAb to C5 or with purified C5a. Treatment with the C5a receptor antagonist or with anti-C5 did not significantly reduce chemokine production (A and B). Cells treated with the C3a receptor antagonist, however, demonstrated reduced levels of both chemokines, so the experiment was repeated after pretreating the cells with 50 μM C3a receptor antagonist or inhibiting factor B with 1379 (C and D). Both treatments resulted in significant reductions in the levels of the chemokines. This small molecule (SB290157) may have agonist activity in some systems, but treatment of the cells with this agent alone did not cause elaboration of the chemokines (E).

of these chemokines by the PTECs. Previous studies have also identified activation of the NF-κB pathway as necessary for the production of IL-8, the human analog of MIP-2, by epithelial cells (33, 34).

Discussion

Renal I/R results in an intense inflammatory response characterized by complement deposition, neutrophil infiltration, and the

generation of proinflammatory cytokines and chemokines. The mechanisms by which the host recognizes injury and initiates the systemic inflammatory response have not been fully elucidated, however. Activation of the alternative pathway is an early event after I/R (20), and complement activation appears to mediate neutrophil infiltration of the posts ischemic kidney (2, 3, 6). Because the complement system is one of the major mechanisms by which the

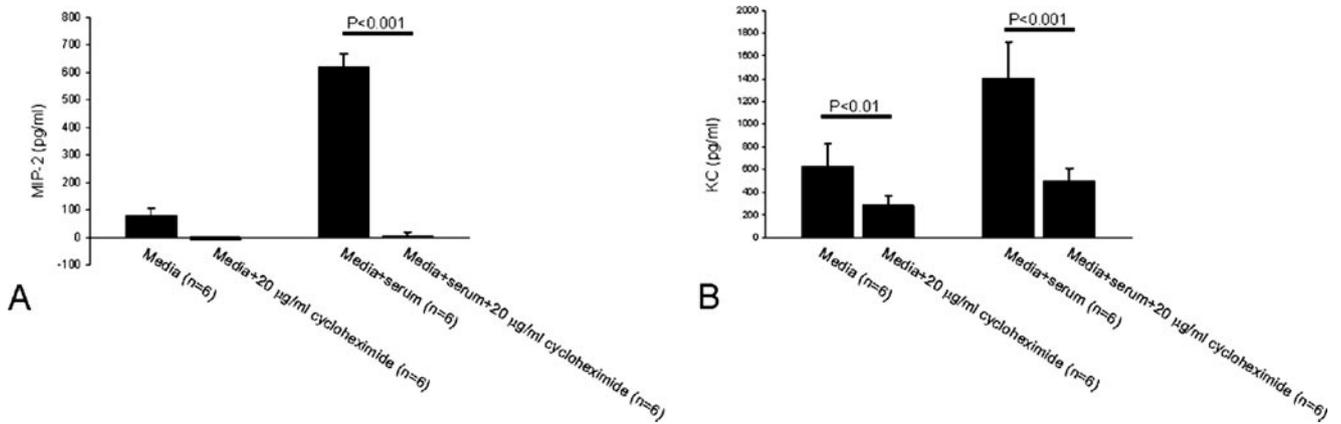


FIGURE 8. Production of MIP-2 and KC by tubular epithelial cells in response to complement activation occurs by de novo synthesis of the proteins. To determine whether production of MIP-2 and KC might, in part, be due to the release of pre-existing stores of the proteins, cells were pretreated with cycloheximide (20 µg/ml) to prevent new protein synthesis. MIP-2 and KC in the supernatants were then measured by ELISA. Concentrations were determined by comparison with a standard curve, and negative values probably represent values below the level of detection for this assay. In cells pretreated for 1 h with cycloheximide, levels of the MIP-2 (A) and KC (B) did not increase in response to an intact alternative pathway indicating that tubular epithelial cells synthesize these factors in response to alternative pathway activation.

body detects injury to self cells, we investigated whether alternative pathway activation triggers the systemic inflammatory response to hypoxic cellular injury.

Gene array analysis initially identified the C-X-C chemokines MIP-2 and KC as factors whose mRNA levels within the kidney increase in a complement-dependent fashion after I/R, suggesting that complement activation mediates tissue inflammation through the generation of these factors. We validated the results of the gene array experiment with quantitative real-time PCR, and using in situ hybridization we identified epithelial cells as a source of these factors within the kidney. Using a PTEC cell line we also found that PTECs in vitro synthesize and secrete both MIP-2 and KC when exposed to an intact alternative pathway. Production of these

chemokines in this in vitro system is mediated by the C3a receptor, but we could not demonstrate a role for the MAC. C5a did stimulate KC production, but C5a antagonism or treatment with the anti-C5 Ab did not prevent KC production. Parallel pathways appear to be activated by serum, therefore, and signaling through the C5aR is not critical. C3a has previously been shown to induce production of IL-8 in endothelial (35) and epithelial (36) cells. Further, activation of the C3a receptor has been shown to result in ERK activation (35) and NF-κB activation (37) in HUVECs and HeLa cells, respectively. In the current study, we found that inhibition of the NF-κB pathway also prevented full production of these factors by PTECs, although production of the chemokines appeared to be independent of the ERK 1/2, JNK, and P38 pathways.

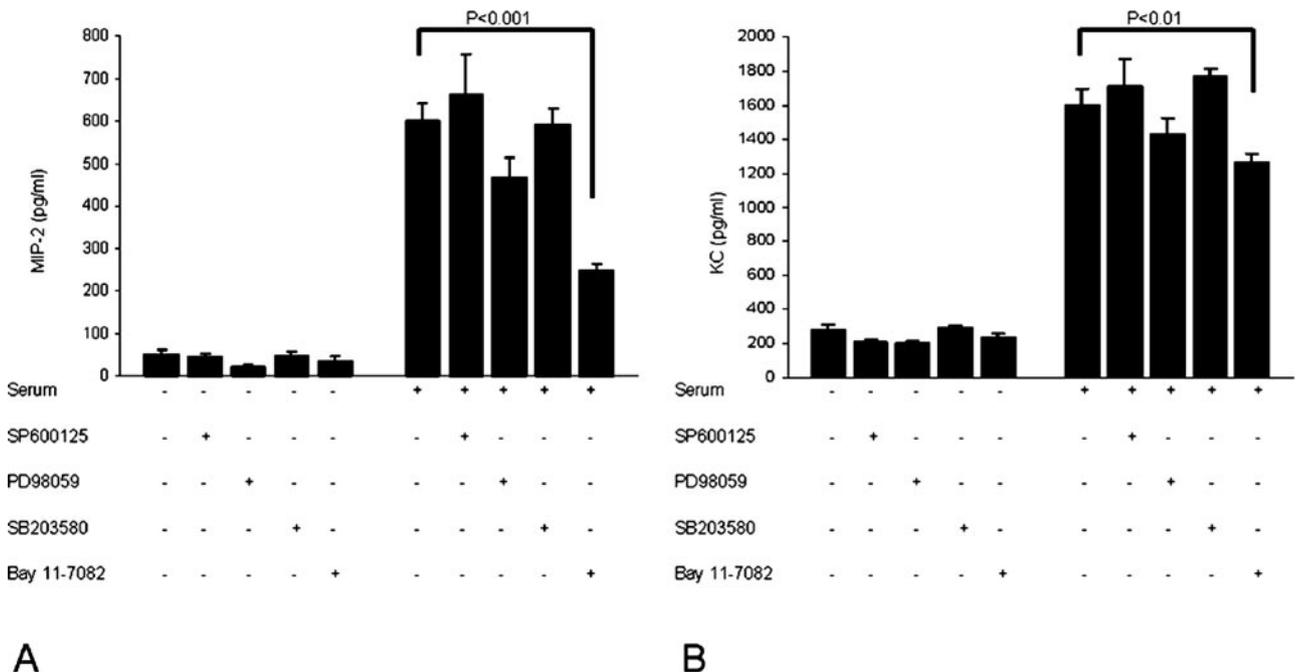


FIGURE 9. Production of MIP-2 and KC by tubular epithelial cells requires activation of the NF-κB pathway. Tubular epithelial cells were treated with inhibitors of the ERK 1/2 (50 µM PD98059), JNK (10 µM SP600125), P38 (10 µM SB203580), and the NF-κB (10 µM Bay11-708) pathways. Inhibition of the NF-κB pathway significantly attenuated the release of both MIP-2 (A) and KC (B) in response to serum with an intact alternative pathway.

Our initial array analysis did not identify complement-dependent changes in the other chemokines evaluated. Because that analysis examined the levels of mRNA, it is possible that other chemokines are modulated by complement activation, but that control is at the level of translation or release of preformed protein. We also looked at a limited number of time-points, so it is certainly possible that genes whose transcription appeared unchanged after 8 h are increased at later time-points. Complement activation is an early event after I/R (20), however, and we focused on time-points within a few hours of evident alternative pathway activation as this is the period during which the acute inflammatory response is generated.

The C3a receptor is expressed in tubular epithelial cells as well as in glomerular epithelial cells (38), and previous studies have demonstrated that C3a can induce C-X-C chemokine production by epithelial cells (36). IL-1 α and TNF- α also induce PTECs to produce IL-8 (39), and exposure of the apical surface of PTECs to albumin induces IL-8 production through NF- κ B activation (34). The similar response of PTECs to these other factors may explain why inhibition of the alternative pathway did not completely abolish production of the chemokines in our in vitro studies, where the cells were likely exposed to other stimuli in the serum.

We have recently demonstrated that hypoxia of PTECs alters the expression of alternative pathway inhibitors on their surface and induces synthesis of C3 by the cells (20). These phenotypic changes favor complement activation on the PTEC surface, a mechanism whereby the body recognizes injury to the cells. The complement system becomes activated after ischemia to several other organs too, including the heart (40), the intestine (41, 42), and skeletal muscle (43). Activation in those tissues, however, involves early classical pathway components. Complement activation after intestinal I/R, for example, occurs as a result of natural IgM which binds to the postischemic tissue (41, 42), whereas activation in the postischemic kidney does not require C4 or the classical pathway (3). It appears, therefore, that the complement system is activated by ischemia in a number of different tissues, but by distinct mechanisms of initiation. The current study suggests that the alternative pathway interacts with the injured renal tubules to generate proinflammatory mediators. Future studies may clarify whether the distinct mechanisms of complement activation in other tissues are also associated with a distinct downstream response of the tissue to the complement activation fragments.

Activation of the alternative pathway then induces the PTECs to produce MIP-2 and KC, thus linking the recognition of tubular epithelial injury to an inflammatory response (44). There is evidence that the toll-like receptors also mediate chemokine production and inflammation after renal I/R, perhaps in response to the release of heat shock proteins which are endogenous ligands of these receptors (45, 46). Furthermore, IL-8 is also produced in the kidney in response to ascending urinary infection (47), and MIP-2 production is increased in a model of cisplatin nephrotoxicity (48). Production of these C-X-C chemokines by the kidney may, therefore, be a general response to tubular injury, although the role of complement activation in these other models has not been investigated.

MIP-2 and KC are potent neutrophil chemotaxins, and are regarded as mediators of the acute inflammatory response to injury (49). In human renal allografts, the level of urinary IL-8 correlated with sustained ARF which was presumably due to ischemic injury at the time of transplantation (50). In another report, urinary levels of GRO- α , the human analog of KC, were higher in transplant recipients with delayed graft function than in those with prompt graft function (16). The authors also reported that these patients had evidence ATN on renal biopsy. Treatment with neutralizing Abs to these two chemokines (51), an antagonist to their shared

receptor (52), or with complement inhibitors (2, 3) protects mice from ischemic acute renal failure. This suggests that both systems are necessary for the systemic response to tubular injury. Whether expression of both of these chemokines is necessary to cause renal injury has not been specifically evaluated. In some circumstances the function of these factors is redundant (53), necessitating dual antagonism to block their function. Other studies have demonstrated a specific role for MIP-2 (54). These chemokines may also have other functions that are important in the development of ischemic ARF, independent of leukocyte trafficking. For example, they may be important in epithelial repair (55, 56), angiogenesis (49), and they have been implicated in the development of fibrosis (57). Nevertheless, production of both factors by PTECs in response to complement activation appears to occur in tandem, and complement inhibition attenuates the generation of both factors within the postischemic kidney.

In conclusion, we have found that the C-X-C chemokines MIP-2 and KC are synthesized by the kidney after I/R in response to activation of the alternative complement pathway. Synthesis of these factors occurs in PTECs, the primary site of complement deposition. In vitro studies confirmed that PTECs rapidly produce these chemokines in response to complement activation, specifically requiring C3a, and this response is mediated by the NF- κ B pathway. Purified C3a did not induce production of these chemokines, suggesting that it requires another cofactor present in serum. Blockade of the alternative pathway in vivo and in vitro prevents the synthesis and release of these chemokines by the PTECs, and their generation by the PTECs in response to complement activation may be an important downstream mediator of complement-induced injury after renal I/R. Complement activation fragments (2–5) and the C-X-C chemokines (51, 52) have been implicated as important mediators of ischemic ARF. Future experiments should explore whether these factors have distinct roles after I/R, or whether they function in a redundant fashion.

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Disclosures

V.M. Holers is a stock-holding consultant and on the Board of Directors for Taligen Therapeutics Incorporated. J. M. Thurman also holds stock in Taligen Therapeutics.

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